ABSTRACT

Investigation of the Role of *ipp-5* and *lfe-2* in the IP_3 Signaling Pathway in *Caenorhabditis elegans* Ovulation

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C. elegans provides a useful system with which to study signaling pathways, giving information that can be applied to many organisms. The use of RNA interference can be used to study the genes involved in many different pathways, including the inositol triphosphate (IP_3) signaling pathway. This pathway is involved in maintaining contractions necessary for *C. elegans* ovulation. Using an RNAi feeding protocol, expression of several protein synthesis and cell signaling genes were knocked down, causing sterility in the wild-type worm. Sterility was rescued in mutants having a constitutively active IP_3 receptor (*itr-1 (sy290)*), revealing that the genes were somehow involved in IP_3 signaling. Use of worms with mutant backgrounds of *ipp-5* and *lfe-2*, which are involved in negatively regulating IP_3, showed that *lfe-2* expression is solely in the spermatheca. When RNAi of ribosomal genes is performed in this mutant, sterility is able to be rescued.

Investigation of the Role of *ipp-5* and *lfe-2* in the IP_3 Signaling Pathway in *Caenorhabditis elegans* Ovulation

by

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A Thesis

Approved by the Department of Biology

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Master of Science

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Accepted by the Graduate School
May 2010

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ACKNOWLEDGMENTS

I would like to take this opportunity to thank all of the people who helped me through the past couple of years which allowed me to complete my thesis. Most importantly, I would like to thank my mentor, Dr. Myeongwoo Lee, who guided me through this process. Your willingness to help and devotion of your time are greatly appreciated. I am very thankful to have been able to work with you and learn from you in your lab over the past two years.

I am also very thankful to the other members of my advisory committee, Dr. Bryan Gibbon and Dr. Robert Kane. Thank you both for your willingness to serve on my committee.

I would also like to thank Jonathan Miles for allowing me to carry on his research in the Lee lab. Your guidance is greatly appreciated as well. I would also like to thank the other members of the Lee lab for their companionship over the past two years, without whom it would have been much more difficult.

To my soon-to-be husband and in-laws, thank you for putting up with me over the past two years as I worked towards my goal. I know that it was not easy. I would also like to thank Danielle for her never ending encouragement. You are an amazing friend and I am blessed to have gotten to know you over these two years. Finally and most importantly, I would like to thank my family for all of their support and love over the past 25 years. Without you I would not have accomplished everything that I have.

DEDICATION

To my family,

for your undying love and support

CHAPTER ONE

Introduction and Background

Caenorhabditis elegans

Caenorhabditis elegans (Figure 1.1) is a model organism used to study several aspects of biology, including genetic analyses. Not only is the organism small (1mm in length) and easily grown (agar plates or liquid culture), but it also has a transparent body which simplifies phenotypic characterization. The nematode's generation time is short, giving rise to adult worms in approximately three and a half days through either self fertilization for the hermaphrodites or sexual reproduction between the male and hermaphrodite. The life cycle includes four larval stages (termed L1 through L4) and an adult stage (Brenner 1974). The hermaphrodite can produce nearly 300 progeny through self fertilization and up to 1400 progeny by mating with the males. It has been found that *C. elegans* has about 1000 somatic cells with the lineage being completely mapped out from zygote to adult stages (Sulston and Horvitz 1977; Wood 1988). Kimble (1981) used laser ablation to show the developmental purpose of specific cells, which was possible because of the previously mentioned, transparent body of the worm.

C. elegans has a relatively small genome comprising approximately one hundred megabases that has been completely sequenced (C. elegans Sequencing Consortium 1998). With this information, molecular techniques like RNA mediated interference (RNAi) and microinjection simplify studies of gene function and signaling pathways during development.

Use of *C. elegans* as a model organism is also beneficial because about 83% of its protein sequences are orthologous to protein sequences found in humans. Therefore, study of these similar proteins could give insight into the structure and function of their human orthologs (Lai and others 2000).

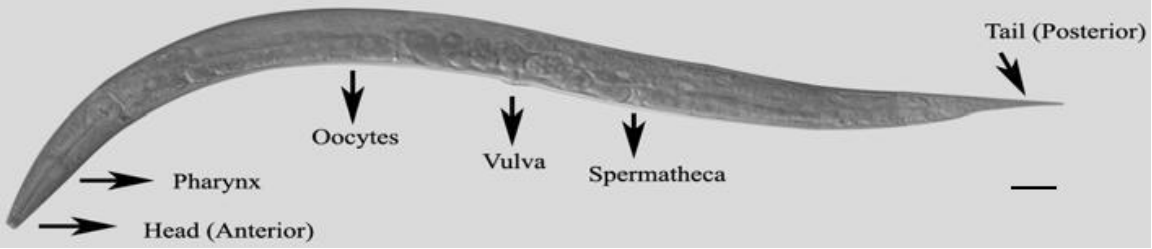

Figure 1.1. DIC image of adult hermaphrodite (magnification 400X) black bar = 40µm

C. elegans Gonad and Ovulation

The *C. elegans* hermaphrodite has two U-shaped, tubular arms that each connect to a spermatheca at their proximal ends (Figure 1.3). These spermathecae then connect to a central uterus. Distal tip cells lead the gonads in the formation of their U-shapes during development. In the distal end of the gonad, oocyte precursor cells are produced and undergo oogenesis as they move proximally. As the oocytes reach the proximal end of the gonad, they line up in a single row and are encapsulated by myoepithelial sheath cells. There are five pairs of these sheath cells around each gonad arm which help maintain the structure of the gonad as well as push oocytes into the spermatheca during ovulation. The sheath cells are made up of non-striated and smooth muscle and contain actin and myosin filaments for contraction. (Strome 1986) These sheath cells are able to uphold the shape of the

maturing oocytes as well as produce contractions which eventually push the oocyte into the spermatheca (Hall and others 1999; Hubbard and Greenstein 2000).

Germ cells occupy the distal portion of the gonad arms and multiply by mitosis. In the distal gonads, the germ cells are kept in mitosis by secretion of LAG-2/DELTA protein from the distal tip cells (DTC). The LAG-2/DELTA binds to its receptor, GLP-1/NOTCH on the germ cells and keeps the cells from entering meiosis. Interaction between LAG-2 and GLP-1 promotes the proliferation of the germ cells in the distal gonad arm through mitosis (Henderson and others 1994; Praitis and others 2001). As the germ cells move away from the mitotic region, they begin to go through the pachytene stage of prophase I of meiosis. Mitogen activated kinase (MAPK) signaling is necessary for germ cells to go through the pachytene stage of prophase I (Church and others 1995). As the cells move further away from the DTC, there is a decrease in the amount of GLP-1 on the cells and they begin oogenesis (Crittenden and others 1994). As the oocytes reach the proximal gonad, they receive signals through the RAS/MAP kinase pathway, which allows the cells to exit pachytene and enter diakinesis. The oocytes will arrest at the diakinesis stage of prophase I until they mature (McCarter and others 1997). Maturation of an oocyte involves several morphological changes that include nuclear migration, nuclear envelope break down (NEBD), and cell rounding. There are many different interactions that eventually signal the oocyte to mature and be ovulated (McCarter

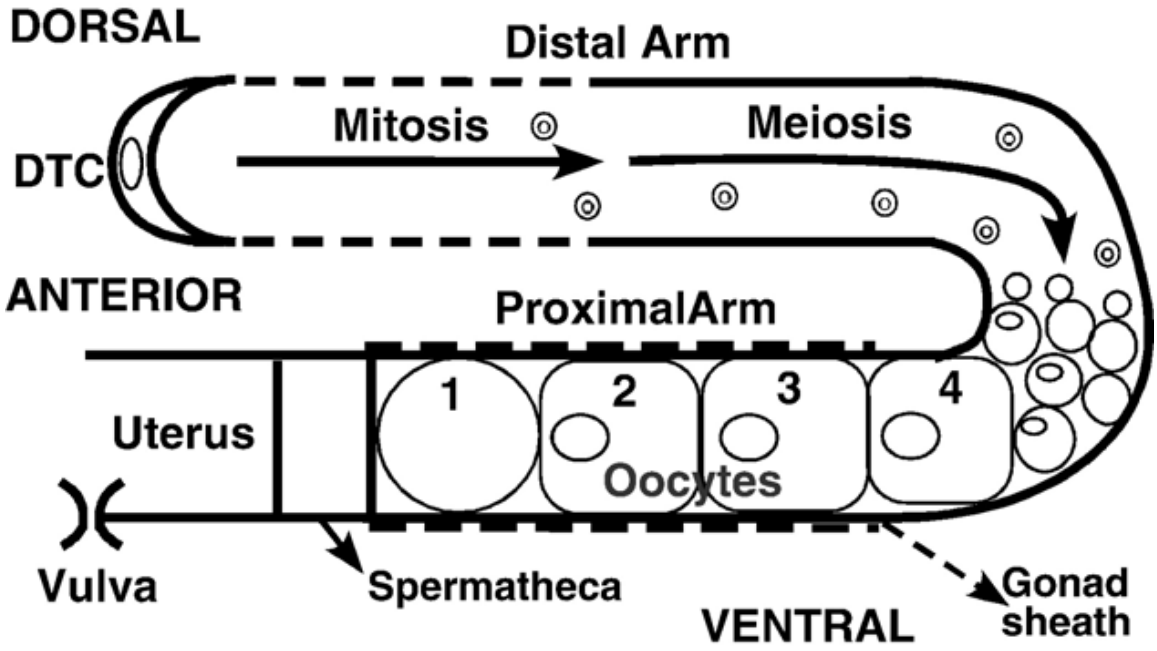

Figure 1.2. Gonad structure of C. elegans. Developing germ cells fill the distal arm. Near the distal tip cells (DTC), germ cells undergo mitosis then become meiotic by leaving the mitotic area. In the proximal arm, matured oocytes are arranged in a row. Oocyte 1 at the front undergoes cell rounding and nuclear disappearance and becomes ready for fertilization and cell division. The oocyte will then advance to the spermatheca for fertilization. A few minutes later, maturation and ovulation of oocyte 2 will follow (Xu and others 2006).

and others 1999). One such interaction involves a major sperm protein (MSP-1) which is released from sperm and binds to a receptor on the oocytes, VAB-1. Binding of MSP-1 to VAB-1 helps the oocytes to mature and promotes ovulation (Miller and others 2001). Another example is the interaction between the sheath cells and the extracellular matrix (ECM). This interaction assists in the organization of the gonad sheath cytoskeletal structure as well as in ovulation in *C. elegans* (Xu and others 2005; Xu and others 2006).

Once the oocyte has matured, interaction between the oocyte and the gonad is mediated by LIN-3/LET-23(Clandinin and others 1998). LIN-3 is initially a transmembrane protein on the oocyte that is excised for the purpose of binding to LET-23, found on the gonadal sheath cell (Liu and others 1999). This interaction between the oocyte and the sheath cells allows an increase in contractions through the IP_3 signaling pathway (Clandinin and others 1998).

Inositol 1,4,5-trisphosphate (IP_3) Signaling Pathway

IP_3 signaling is the main signaling pathway involved in ovulation of the *C. elegans* oocyte into the spermatheca. The pathway generates sheath cell contractions by releasing the calcium ions stored in the endoplasmic reticulum (Clandinin and others 1998). In the sheath cells are receptor tyrosine kinases which activate phopholipases. These phopholipases can then generate molecules of IP_3 (Berridge 1993).

When the LIN-3/epidermal growth factor (EGF) protein, produced in the *C. elegans* oocyte, is secreted, it binds to and activates its receptor, LET-23/epidermal growth factor receptor (EGFR), on the sheath cells. This will then activate the

phospholipases necessary for production of IP_3. This ensures that the oocyte is indeed mature and ready for ovulation before the sheath cell contractions begin (Yin and others 2004)(Figure 1.4).

IP_3 production occurs because of the hydrolysis of phophotidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and IP_3 by phospholipase C (PLC) (Yin and others 2004). There are several genes in *C. elegans* that encode phospholipases, but only *plc-1* and *plc-3* have been shown to have any effect on fertility. Studies have shown that expression of PLC-3 is mainly in the sheath cells and the spermatheca (Yin and others 2004). Once IP_3 is produced through hydrolysis of PIP_2, there are negative regulations to manage the rate of sheath cell contractions. IPP-5 and LFE-2 are enzymes that transform IP_3 into structures that are unable to bind to its ITR-1 receptor (Bui and Sternberg 2002). *lfe-2* encodes an IP_3 kinase that generates inositol tetrakisphosphate (IP_4) and *ipp-5* encodes a type I 5'-phosphatase that produces inositol bisphosphate (IP_2) (Bui and Sternberg 2002). Both of these forms, IP_4 and IP_2, are unable to bind to the ITR-1 receptor located on the endoplasmic reticulum and decrease the amount of IP_3 available in the cell. Mutants with deletions in *ipp-5* gene show a defect in the closing of the spermatheca and allow multiple oocytes to be ovulated at a time (Bui and Sternberg 2002). These negative regulators ensure that the right amount of IP_3 is available in the cell to trigger its receptor, ITR-1, to release the appropriate amount of calcium ions into the cytoplasm in order to generate the necessary sheath cell contractions for ovulation (Clandinin and others 1998).

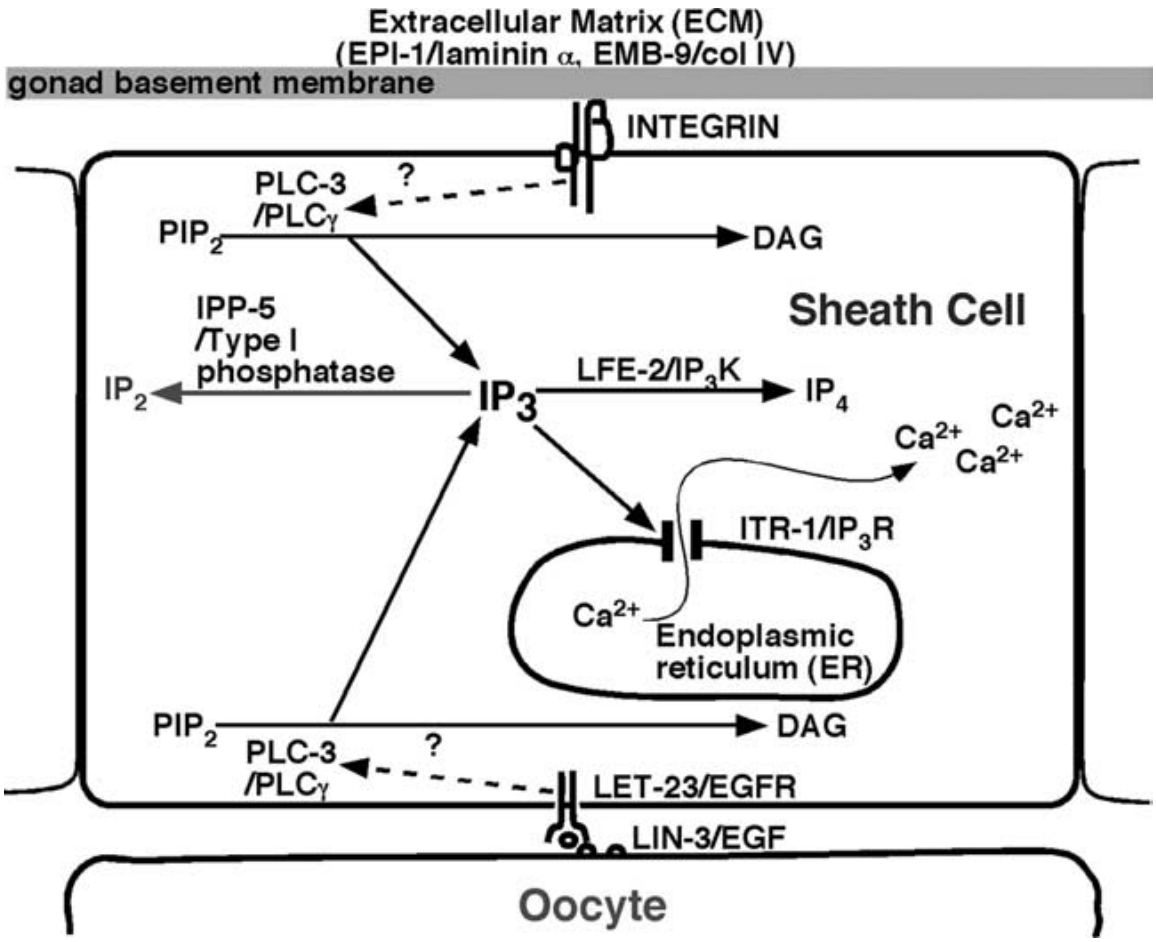

Figure 1.3. Sheath cell of proximal gonad. The signaling pathway necessary to generate sheath cell contractions. The oocyte signals the sheath cell and activates the phopholipase to generate IP_3. IP_3 then binds to its receptor, ITR-1, on the endoplasmic reticulum to release calcium stores and increase contraction of sheath cells (Xu and others 2005).

The release of calcium ions from the smooth ER is responsible for the ovulatory sheath cell contractions. The signaling of the IP_3 pathway ensures that there is a mature oocyte ready for ovulation before the ovulatory contractions begin and before the spermatheca can dilate (McCarter and others 1999). Sperm assist in maintaining basal sheath contractions, which occur at a rate of seven to eight times per minute, but they do not help in ovulatory sheath contractions (Miller and others 2001). Once a matured oocyte is present in the proximal gonad, the ovulatory sheath contractions begin, which increases to 18 to 22 times per minute (McCarter and others 1999; Yin and others 2004).

RNA Interference and Mechanism

RNA interference is a response to the presence of double stranded RNA (dsRNA) in the cell. dsRNA is rarely seen in the cell, with the exception being viruses which sometimes use it as part of their replication process (Blevins and others 2006). The presence of dsRNA has shown to cause an immune response involving interferons in many animals, including mammals (Reynolds and others 2006) which could allow for the development of new gene therapies (Shuey, McCallus, Giordano 2002).

Some of the first uses of RNAi involved microinjection of the dsRNA into the embryo of the nematode, allowing the embryo itself to be subject to the dsRNA and the resulting interference (Fire and others 1998). In these early experiments, the dsRNA was shown to be able to cross cellular boundaries, allowing for a complete systemic knockdown of the target RNA. For this to be accomplished, the mRNA produced in the organism needs to be at a relatively high expression level,

otherwise it could be overlooked by the complementary dsRNA and escape the mRNA degradation complex (Fire and others 1998).

In the RNAi process, dsRNA are typically cleaved into shorter pieces that are close to 21-22 nucleotides in length. If the dsRNAs are not at the appropriate length, the protein DCR-1, or Dicer, will cut the dsRNA into these shorter pieces. Dicer is an RNase III nuclease that has been shown to be able to cut dsRNAs (Ketting and others 2001). Dicer will cut these dsRNAs into shorter, more appropriate lengths if they are longer than is optimal (Ketting and others 2001; Bernstein and others 2001). Once they are the right length, the small interfering RNAs (siRNAs) will be incorporated into the RNA-induced silencing complex, or RISC. The RISC uses the siRNAs as templates for the degradation of complementary mRNAs that naturally occur in the cell (Bernstein and others 2001)(Figure 1.5).

In *C. elegans*, there are pores that are specific for the transport of dsRNA. The genes that encode these specific pores are *sid-1* and *sid-2* (Feinberg and Hunter 2003; Winston and others 2007). Because of these pores, the animal is able to have a systemic response to the dsRNA producing a complete knockout. The only cells in *C. elegans* that seem to be able to resist the effects of RNAi are the neurons (Krichevsky and Kosik 2002).

Using the microinjection method, initially used by Fire et. al. 1998, to introduce dsRNA is one of the more difficult methods. A feeding method was made available by the production of a bacterial library that included a great majority of the *C. elegans* genome (Kamath and others 2003). The library was produced using

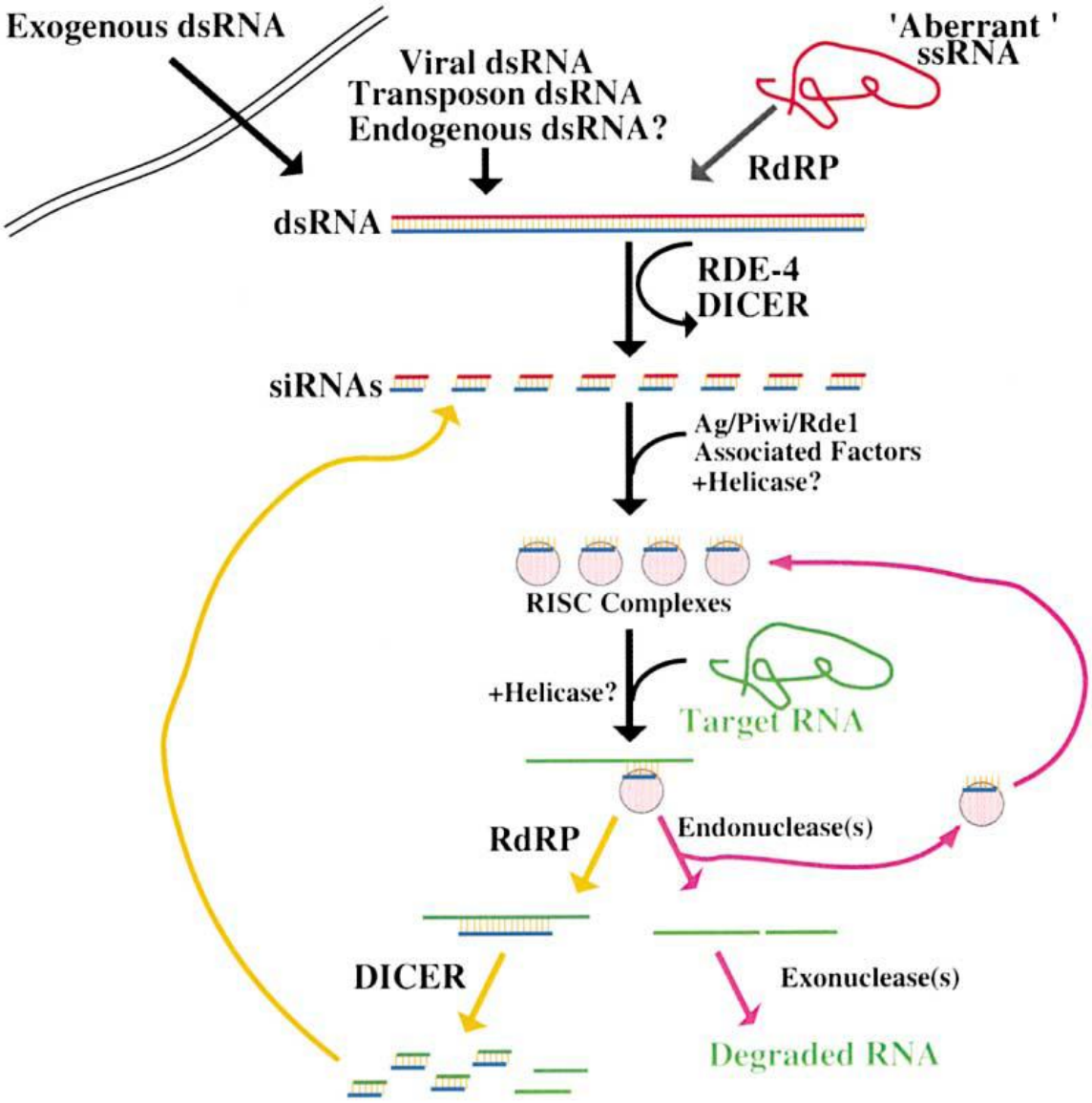

Figure 1.4. Diagram of RNAi mechanism. Double stranded RNA is digested by Dicer which produces siRNA molecules. These will enter the RISC to be used as templates for degradation of the complementary mRNA (Sijen and others 2001).

the L4440 vector, which has T7 promoters on both sides of the multiple cloning site. These T7 promoters allowed for the production of complementary strands of RNA, making the dsRNA necessary for RNAi (Timmons, Court, Fire 2001)(Figure 1.6). These plasmids were then transformed into HT115(DE3), an *Escherichia coli* that does not have the activity of RNase III, which degrades dsRNA (Timmons, Court, Fire 2001). These *E. coli* bacteria are able to produce T7 polymerase when in the presence of lactose, thereby only inducing the expression of dsRNA when wanted (Timmons, Court, Fire 2001).

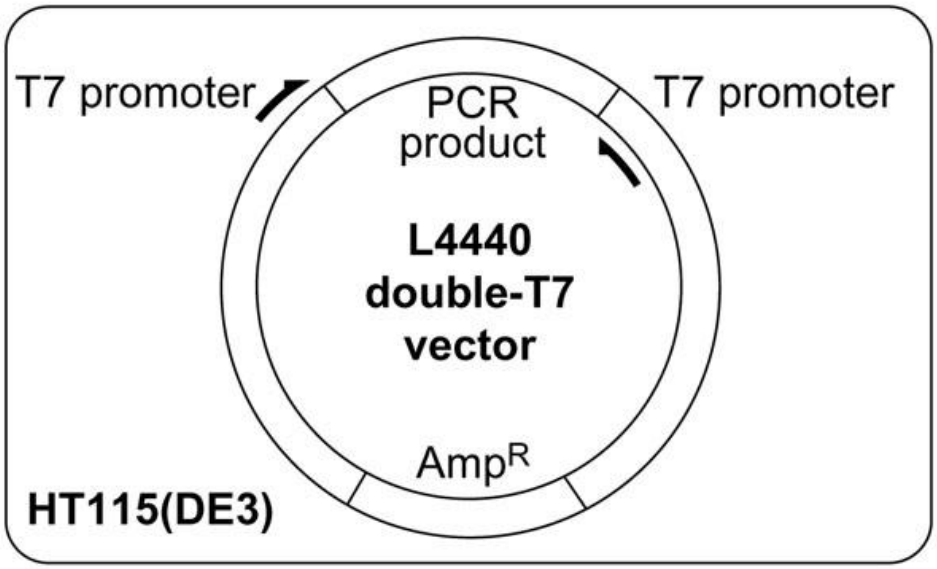

Figure 1.5. The plasmid construct L4440 within an HT115(DE3) bacteria. The plasmid contains the gene of interest between the dual T7 promoters. The dual promoters will create the complementary dsRNA (Kamath and others 2001).

There are a handful of methods that can be used to introduce dsRNA into *C.elegans*, including the use of microinjection which was the primary technique used initially (Fire and others 1998). The microinjection method is still used in some cases, but is one of the more difficult because of the small number of worms that can

be used and the possibility of needing a specific injection site. The individual worms have to be paralyzed and then individually injected with the dsRNA, which would take enormous amounts of time for larger sample sizes (Fire and others 1998).

Since *C. elegans* has the ability for dsRNA to cross cell boundaries, it has made the use of other, easier methods of RNAi available. One of these methods is called the soaking method, in which the worms are soaked in a liquid medium of lipid drops containing dsRNA (Maeda and others 2001). In this method, the worms are soaked in a dsRNA solution for 24 hours, during which time they soak up the dsRNA (Maeda and others 2001). After this time, they are placed on NGM plates seeded with normal OP50 bacteria. Since the worms have taken up the dsRNA while in the solution, the RISC proteins can be initiated and the dsRNA can spread throughout the body of the animal though the pores generated by *sid-1*. The soaking method allows for larger samples to be introduced to the dsRNA, however this method does not show the potency that the microinjection and feeding methods have shown (Tabara, Grishok, Mello 1998).

The final protocol available for the introduction of dsRNA into *C. elegans* is the feeding protocol, which is the simplest of the three. *C. elegans* eat bacteria, like the HT115 *E. coli* used in production of the dsRNA, and break them down in their pharynx. As this food moves through the intestines, the nutrients are taken up as well as the dsRNA (Timmons and Fire 1998). This protocol also allows for larger sample sizes, making it the easiest protocol for induction of RNAi in *C. elegans*.

Specific Aim: Analysis of the Role of ipp-5 and lfe-2 in the IP_3 Pathway and in Sterility

When studying the IP_3 signaling pathway, a great model system to use is the *C. elegans* gonad and ovulation. Sterility was seen in both the wild type (N2) worm and the *ipp-5* mutant, while sterility was rescued in both the *itr-1 (sy290)* and *lfe-2* mutants. The *itr-1 (sy290)* mutant has a gain-of-function mutation which constitutively activates the IP_3 receptor on the endoplasmic reticulum. Using the feeding method of RNAi, the IP_3 signaling pathway was looked at more closely. Use of several genes, which had previously been found to cause sterility in the N2 background but were rescued by the *itr-1* gain-of-function mutant, were examined in the *ipp-5* and *lfe-2* mutant backgrounds. Many of these genes encode for ribosomal or proteasomal proteins, but also included some structural proteins as well. Many of these genes seem to play an important role in the ovulation of *C. elegans* and may interact with the IP_3 signaling pathway.

CHAPTER TWO

Materials and Methods

Identification and Analysis of RNAi Induced Sterility

Preparation of Laboratory Reagents and Plates

M9 buffer solution. This solution is used with *C. elegans* in many different procedures, for example, washing worms off of plates. It was prepared by adding together 3 mg/mL of KH_2PO_4 (EMD Chemicals, NJ, USA), 6 mg/mL of Na_2HPO_4 (Fischer Scientific, NJ, USA), and 5 mg/mL of NaCl (EM Science, NJ, USA) in 1L of DI water. After autoclaving for 15 minutes, 0.12 mg/mL of 1 M $MgSO_4$ was added to the solution after it was cooled. M9 buffer was stored at room temperature until needed.

Nematode freezing solution. A special solution is used for freezing of worm strains. About 1L of solution was made by combining 5.85 g of NaCl (EM Science, NJ, USA), 6.8 g of KH_2PO_4 (EM Science, NJ, USA), 300 g or 240 mL of glycerol (EM Science, NJ, USA) and 5.6 mL of NaOH (1 M, EM Science, NJ, USA). The volume was brought up to 1 L by adding DI water. The solution was then autoclaved for 15 minutes and allowed to cool. Once cool, 3 mL of sterile 0.1 M $MgSO_4$ was added and the solution was stored at room temperature.

β-lactose stock solution. Lactose is used to induce the production of dsRNA in the RNAi bacterial lawns. When the RNAi plates were prepared, a 20% w/v solution

of β-lactose was added after autoclaving. To preparethe stock solution, 10 g of lactose powder (Becton and Dickinson, MD, USA) was added to 50 mL of DI water in a 100 mL beaker. The beaker was placed on a hot plate after adding a magnetic stir bar to help dissolve the powder. Once the lactose was completely dissolved, the solution was passed through a sterile filter (0.22 micron pore size) to guarantee the solution was sterile. The filtrate was transferred into a sterile 50 mL centrifuge tube and aliquoted into sterile 15 mL centrifuge tubes, 4.5 mL to each tube. These 15 mL tubes were then stored at -20° C until needed.

Ampicillin stock solution. The RNAi bacteria used contain a gene that confers resistance to the antibiotic ampicillin. To ensure that only the bacteria of interest was grown on the RNAi plates, ampicillin stock solution was added after autoclaving. The stock solution was prepared by weighing out 2 g of ampicillin (Shelton Scientific, CT, USA) and adding it to 50 mL of DI water in a 100 mL beaker. After dissolving, the solution was passed through a sterile filter (0.22 micron pore size) to ensure its sterility. The filtrate was transferred to a sterile 50 mL centrifuge tube and then aliquoted into 15 mL sterile centrifuge tubes, with 4.5 mL to each tube. These tubes were stored at -20° C until needed.

RNA-mediated interference (RNAi) plates. Special plates were used in the growth of the RNAi bacteria to produce dsRNA. Different volumes can be used, but usually four (4) liters were prepared at a time. In a sterile plastic weighing dish, 68 g of agar, 10 g of peptone, and 12 g of NaCl were weighed out. These were mixed

with 4 L of DI water in a 6 L Erlenmeyer flask along with a magnetic stir bar and then autoclaved.

After autoclaving, the mixture was allowed to cool to approximately 55°C. Once cooled, 4 mL each of cholesterol, sterile magnesium sulfate, and sterile calcium chloride were added to the mixture. In addition, 100 mL of sterile 1 M KH_2PO_4 buffer (86.7 mL of 1M KH_2PO_4 and 13.3 mL of 1M K_2HPO_4) was added to the agar solution. To induce the production of T7 polymerase in the bacteria, 4.5 mL of a 20% lactose solution was added to the mixture. The bacteria used, HT115(DE3), has shown that when plated on agar with a concentration of approximately 1mM of IPTG, there is optimal production of dsRNA (Kamath and others 2001). Ampicillin was also added (4.5 mL) to the plates to prevent contamination, with a final concentration of 40 μg/mL.

Once everything was added to the agar, the plates were poured using a Wheaton Unispense pouring machine. Approximately 13.5 mL was poured into each 13.5 mm plate. The plates were allowed to sit and harden overnight, and were then transferred to plastic boxes and stored at 4°C. Normal nematode growth media (NGM) plates were made in a similar fashion, but excluded the addition of the ampicillin and 20% lactose.

Lysogeny broth (LB). In order to seed the RNAi bacteria onto RNAi plates, the colonies first had to be inoculated and grown in LB media. To prepare the media, 5g of tryptone (Becton and Dickinson, MD, USA), 2.5 g of yeast extract (Becton and Dickinson, MD, USA), 5 g of NaCl, and 10 mL of Tris-HCl at pH 7.5 (Fischer Scientific, NJ, USA) were added to 500 mL of DI water and mixed using a magnetic stir bar.

Once everything was dissolved, the LB was aliquoted into 150 mL bottles, 100 mL in each, and were autoclaved. LB was then stored at 4°C until needed.

In order for the LB to be used to culture RNAi bacteria, ampicillin had to be added. For 100 mL of LB, 100 µL of ampicillin was added. This helped to ensure that only the RNAi bacteria would be growing in the culture.

LB plates. To grow colonies of OP50 stock bacteria, to be inoculated in LB, LB plates were needed. The solution was prepared the same as LB, however agar (7.5 g) was added before autoclaving and the solution was not aliquoted into separate bottles. After autoclaving, the media was poured into 100 mm diameter petri dishes and allowed to sit overnight to solidify. Once solid, the plates were stored at 4°C.

If colonies of RNAi bacteria needed to be grown, ampicillin would have to be added (500 µL) to the LB agar solution after autoclaving. Once the ampicillin is added, then the solution can be poured in the same manner as the normal LB plates.

C. elegans Strains

The following worm strains were used in this study: wild-type N2, *itr-1/lfe-1 (sy290)* IV, *lfe-2 (ps2286)*, *ipp-5 (ps3653)*. All strains were grown and maintained at room temperature (26°C) with *Escherichia coli* OP50 seeded onto NGM agar plates.

The worms were frozen using the standard protocol. To begin, about 3 mL of M9 buffer was used to float L1 and L2 stage larva off of a freshly starved plate. After about 3-5 minutes, the worms were collected using a sterile glass Pasteur pipette and transferred to a 15 mL centrifuge tube. 150 µL of sterile 60% sucrose solution was added and mixed well. Tubes were then centrifuged at 3300 rpm in a Fischer

Scientific Centrific model 228 centrifuge for 5 minutes. After centrifugation, the supernatant was removed from the tube and 500 µL each of M9 buffer and nematode freezing solution were added. Tubes were then gently shaken to ensure mixture of the solutions before aliquoting to cryovials. Cryovials were placed in Styrofoam racks, covered with aluminum foil, and placed in the -80°C freezer. This ensures that the worms freeze at the appropriate pace of -1°C per minute.

RNAi Bacterial Preparations

Bacterial libraries were kept in -80°C and only removed when needed to grow colonies. To grow colonies, a sterile 100 µL pipette tip was used to scrape the solution and was then scraped on an LB/ampicillin agar plate. The plate was placed in a 34°C incubator for 12-14 hours, or overnight. Plates were removed from the incubator, wrapped with parafilm and kept at 4°C until needed.

Bacterial colonies were then isolated from the LB agar plate with a 100 µL pipette tip by scraping the plate and used to inoculate a culture tube with a small amount of LB/ampicillin broth. The LB had ampicillin added to prevent the growth of other, unwanted bacteria. The culture tubes were placed in the 34°C incubator on a shaker for approximately 12-14 hours, or overnight. After this time, the tubes were removed and placed at 4°C for use later that day.

The LB broth was used to seed four (4) RNAi plates. Approximately 50-100 µL of broth was spread onto each RNAi plate using a sterile pipette. The RNAi plates then sat with the agar side down at room temperature for 12-14 hours, or overnight. During this time, the RNAi bacteria could soak into the plate and grow.

The following day, adult *C. elegans* worms, N2, *itr-1 (sy290), ipp-5,* or *lfe-2*, were transferred onto the plates and allowed to lay eggs for several hours. After about four hours, the plates were checked for the presence of eggs (~ 30 per plate) and the adults were killed by flame. Alternatively, the eggs could have been taken directly from the stock plates and plated on the RNAi seeded plates.

The same day that worms/eggs are transferred to RNAi seeded plates, a larger LB/ampicillin broth inoculation was made. The tubes were again placed at 34℃ on a shaker for 12-14 hours, or overnight. After that time, the tubes were removed and placed at 4° C until used later that day.

RNAi plates were removed from the 4° C storage refrigerator to be seeded with RNAi bacteria. The experiments required the use of thirty (30) plates for each of the worm strains used. Since there were four (4) strains (N2, *itr-1 (sy290), ipp-5, lfe*-2) being used, the experiment required 120 RNAi plates. All 120 plates were seeded with the LB/ampicillin culture broth prepared the day before. Once seeded with RNAi bacteria, these plates were allowed to sit, agar side up, for approximately 12-14 hours, or overnight, to allow the bacteria to soak into the plate and grow.

After this time, worms from the RNAi seeded stock plates were transferred to the newly seeded RNAi plates. One worm was placed on each plate, with a total of 120 worms transferred, thirty (30) of each strain, making sure to clearly label the plates with the correct worm strain as well as the RNAi bacteria used. All of these plates were placed in a plastic container and placed in the 25° C incubator for approximately 48 hours. After this time, the worms were scored for sterility.

C. elegans Phenotypic Characterization and Microscopy

Rigid scoring for sterility was used for the worms being observed. Since there was only a single worm transferred to each plate, those plates which contained more than the single worm were counted as fertile. However, if the plate contained only the single, original worm plated, it was counted as sterile. Sterility could be caused by a number of factors including, sheath cell contraction defects, sperm production defects, oocyte production defects, or defects within the spermatheca. Due to these multiple possibilities, microscopy was required for a closer look.

The adult worms grown during the experiments using RNAi were examined more closely using differential interference contrast (DIC) microscopy. To examine the *C. elegans* gonads, the worms were soaked in 20 mM sodium azide (NaN_3) and then placed on 2% agarose pads on thin glass slides. The worms were then examined using a Nikon TE2000-U inverted microscope with a 40X Plan Fluor objective lens. Images of the *C. elegans* gonad were taken with a CoolSnap cf monochrome camera. The oocytes in these images were examined, if present. Many times, if there are sheath cell contraction defects, it will result in an endomitotic oocyte (Emo) phenotype, where the oocyte goes through multiple rounds of replication without cellular division while stuck in the proximal gonad. The oocytes continue to build up in the proximal gonad and become compacted.

In some cases, worms were also treated using 4',6-diamidino-2-phenylindole (DAPI) to more easily visualize the endomitotic (Emo) phenotype. DAPI can easily pass through cellular membranes and binds to DNA. Then, using ultraviolet light, it

will fluoresce to show the location of nuclei. In the worms showing the Emo phenotype, these particular oocytes undergo multiple rounds of nuclear replication without cellular division. This gives more DNA for the DAPI to bind to and makes those nuclei easily visible.

CHAPTER THREE

Results

In previous sterility screens, there were several genes that caused sterility in the wild-type N2 *C. elegans*, but could be rescued in the *itr-1 (sy290)* mutant background, suggesting that these genes play a role in the IP_3 signaling pathway involved in ovulation (Miles unpublished data). Many of the genes that showed this result are involved in protein synthesis.

Protein Synthesis Proteins

mRNAs are processed in the nucleus and then transported to the cytoplasm to be translated into the actual proteins. The process of protein synthesis involves many elements that make up the ribosomes as well as chaperones that fold the proteins after production. The ribosomes are made up of two subunits, a 60S large subunit and a 40S small subunit (Dinman 2009). The nucleolus, in the nucleus, is the center where rRNAs are processed, ribosomes are produced, and a number of other functions are performed (Olson and Dundr 2005). The large ribosomal subunit can be broken down into a 5S rRNA, a 28S rRNA, a 5.8S rRNA, and almost 50 proteins, with the rRNAs acting as catalytic domains for the subunit (Nissen and others 2000; Rodnina and Wintermeyer 2009). On the other hand, the small ribosomal subunit only has an 18S RNA and about 30 proteins (Dinman 2009). There are many other factors that play a role in protein synthesis other than the ribosomes themselves. These include factors involved in initiation, elongation, and

termination of translation (Dinman 2009). With this many components needed for protein synthesis, it is not a surprise that there are negative effects when these proteins are lost.

Previously several genes involved in protein synthesis were knocked out using RNAi showed to have negative effects on the fertility of the wild type *C. elegans* strain, N2 (Miles unpublished data). Many of these genes were able to be almost fully rescued in the mutant background *itr-1 (sy290)*, which may imply a role in the IP_3 pathway. In order to determine if and how these genes play a role in the IP_3 signaling pathway, RNAi was utilized to eliminate these various proteins using the mutant backgrounds of *ipp-5* and *lfe-2*.

Of the protein synthesis genes looked at, four of them were components of the small ribosomal subunit: *rps-7*, *rps-24*, and *rps-28* (Table 3.1). In the case of RNAi using the *rps-7* RNAi, the N2 worms were shown to be sterile 87% (N=30) of the time while worms with the *itr-1 (sy290)* background only showed sterility 13% (N=30)of the time (Figure 3.1). For this same gene, RNAi with the *ipp-5* background were sterile 83% (N=30) of the time while mutants with the *lfe-2* background were only sterile 33% (N=30) of the time. This suggests that the RPS-7 protein may be important in the negative regulation of IP_3 in the signaling pathway.

Just like with *rps-7*, the protein synthesis gene *rps-28*, showed high sterility in both the N2 wild type and the *ipp-5* background but not in the *itr-1 (sy290)* and *lfe-2* mutants (Figure 3.2). When the transcript of *rps-28* was eliminated using RNAi, 80% (N=30) of the N2 worms and 100% (N=30) of the *ipp-5* mutant

Table 3.1. Protein synthesis genes knocked out using RNAi where ≥60% of N2 worms were sterile. N = number of animals.

Locus	Function	Reference	% Sterile (N)			
			N2	*itr-1 (sy290)*	*ipp-5*	*lfe-2*
rps-28	Small 40S ribosomal subunit S28 protein	Wormbase	80 ± 7.3 (30)	13 ± 6.1 (30)	100 (30)	17 ± 6.9 (30)
rps-7	Small 40S ribosomal subunit S7 protein	Wormbase	87 ± 6.5 (30)	13 ± 6.1 (30)	83 ± 6.9 (30)	33 ± 8.6 (30)
rps-24	Small 40S ribosomal subunit S24 protein; Germline development	Wormbase	100 (30)	17 ± 6.9 (30)	100 (60)	95 ± 2.8 (60)
rpl-7A	Large 60S ribosomal subunit L7A protein; SMG-mediated nonsense suppression	Mitrovich 2000	83 ± 6.9 (30)	13 ± 6.1 (30)	97 ± 3.1 (30)	33 ± 8.6 (30)
rpl-36	Large 60S ribosomal subunit L36 protein; Embryonic/germline development	Wormbase	90 ± 5.5 (30)	13 ± 6.1 (30)	100 (30)	7 ± 4.7 (30)
rpl-20	Large 60S ribosomal subunit L18A protein	Wormbase	80 ± 7.3 (30)	10 ± 4.1 (30)	90 ± 5.5 (30)	7 ± 4.7 (30)

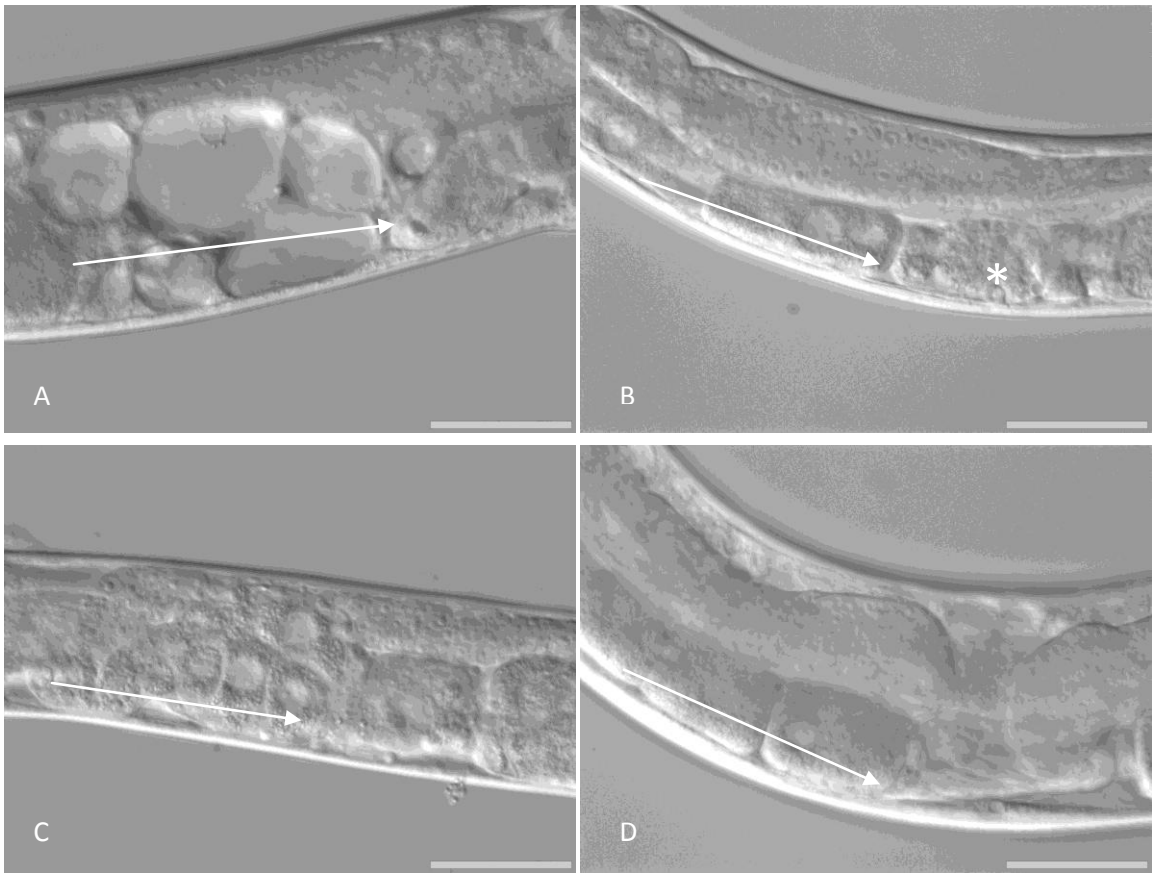

Figure 3.1. Worms fed *rps-7* RNAi bacteria. (A) An N2 worm with compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing rescued sterility with a fertilized egg in utero. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A *lfe-2* worm showing well formed and aligned proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca. Asterisk indicates egg in utero.

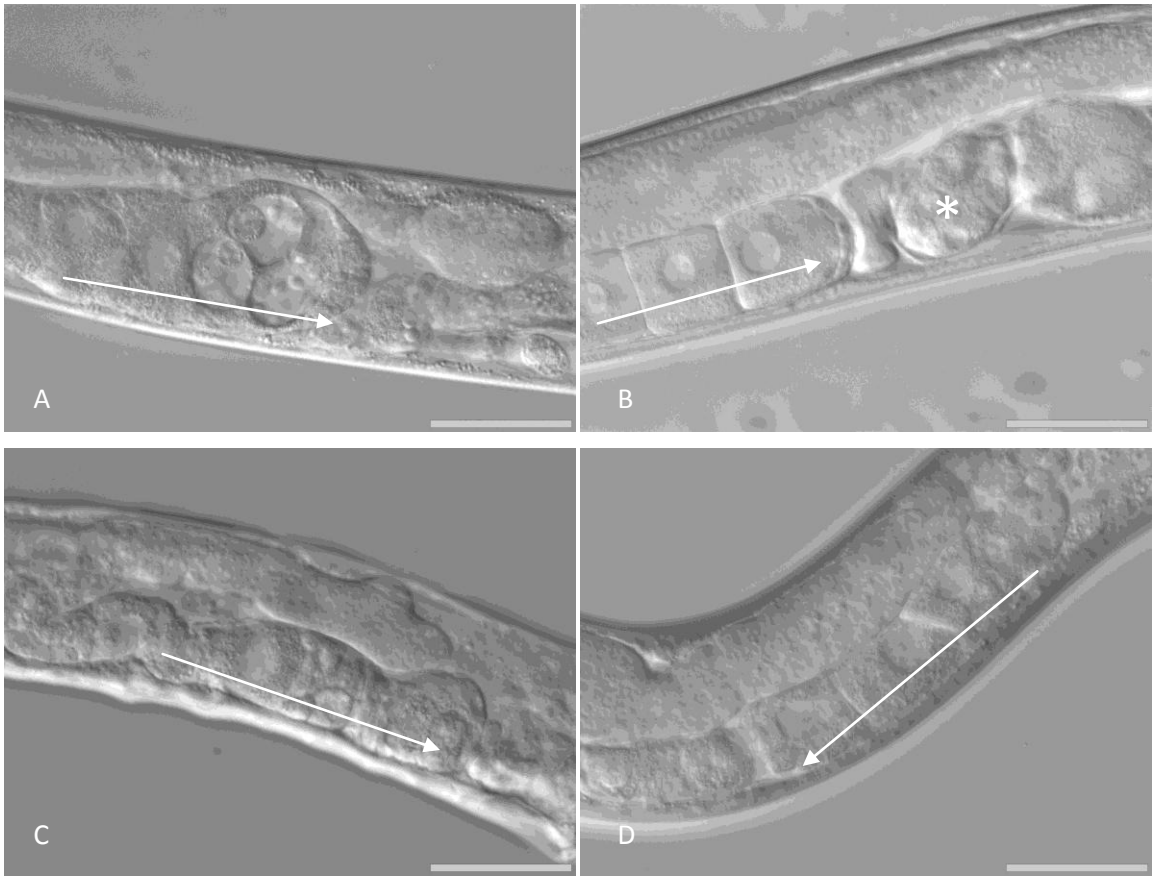

Figure 3.2. Worms fed *rps-28* RNAi bacteria. (A) A DIC image of a wild-type N2 worm with compacted proximal oocytes. (B) A DIC image of an *itr-1 (sy290)* worm showing rescued sterility with a fertilized egg in the uterus. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A DIC image of a *lfe-2* worm showing well formed and aligned proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca. Asterisk indicates egg in utero.

worms were sterile. However, only 13% (N=30) of *itr-1 (sy290)* and 17% (N=30) of *lfe-2* worms appeared to be sterile. This again suggests that there is a possible role for these protein synthesis genes in the negative regulation of IP_3 in its signaling pathway. The two other genes of the small ribosomal subunit did not follow the same pattern as *rps-28* and *rps-7*. When RNAi was induced using bacteria producing dsRNA for *rps-24*, the N2 worms and *ipp-5* mutants again showed a high rate of sterility, but the *lfe-2* mutants did as well, while the *itr-1 (sy290)* worms again showed only 17% (N=30) sterility. Both N2 and *ipp-5* worms showed sterility at 100% (N=30,60 respectively) when *rps-24* translation was knocked down, but *lfe-2* worms were also very high with 95% (N=60) showing sterility. Again, the high sterility rates in the *ipp-5* and here the *lfe-2* mutants suggest a role in the negative regulation of IP_3 and its signaling pathway.

There were also genes involved in protein synthesis, which make up part of the large ribosomal subunit, that showed sterility in the lines used. In the case of *rpl-20*, when its translation was arrested by RNAi, the N2 worms showed sterility 80% (N=30) of the time, while only 10% (N=30) of the *itr-1 (sy290)* mutants were sterile (Figure 3.4). For the same gene, *rpl-20*, the *ipp-5* mutant showed a high sterility at 90% (N=30) and of the *lfe-2* worms, only 7% (N=30) were sterile. Following the same pattern, RNAi of *rpl-7A* left 83% (N=30) of N2 worms sterile and only 13% (N=30) of the worms with an *itr-1 (sy290)* mutant background. RNAi of *rpl-7A* also resulted in the sterility of *ipp-5* mutants 97% (N=30) of the time, while it only left 33% (N=30) of *lfe-2* mutants sterile (Figure 3.5). As with *rpl-20* and *rpl-7A*, production of *rpl-36* dsRNA left the majority (90%, N=30) of N2 worms sterile

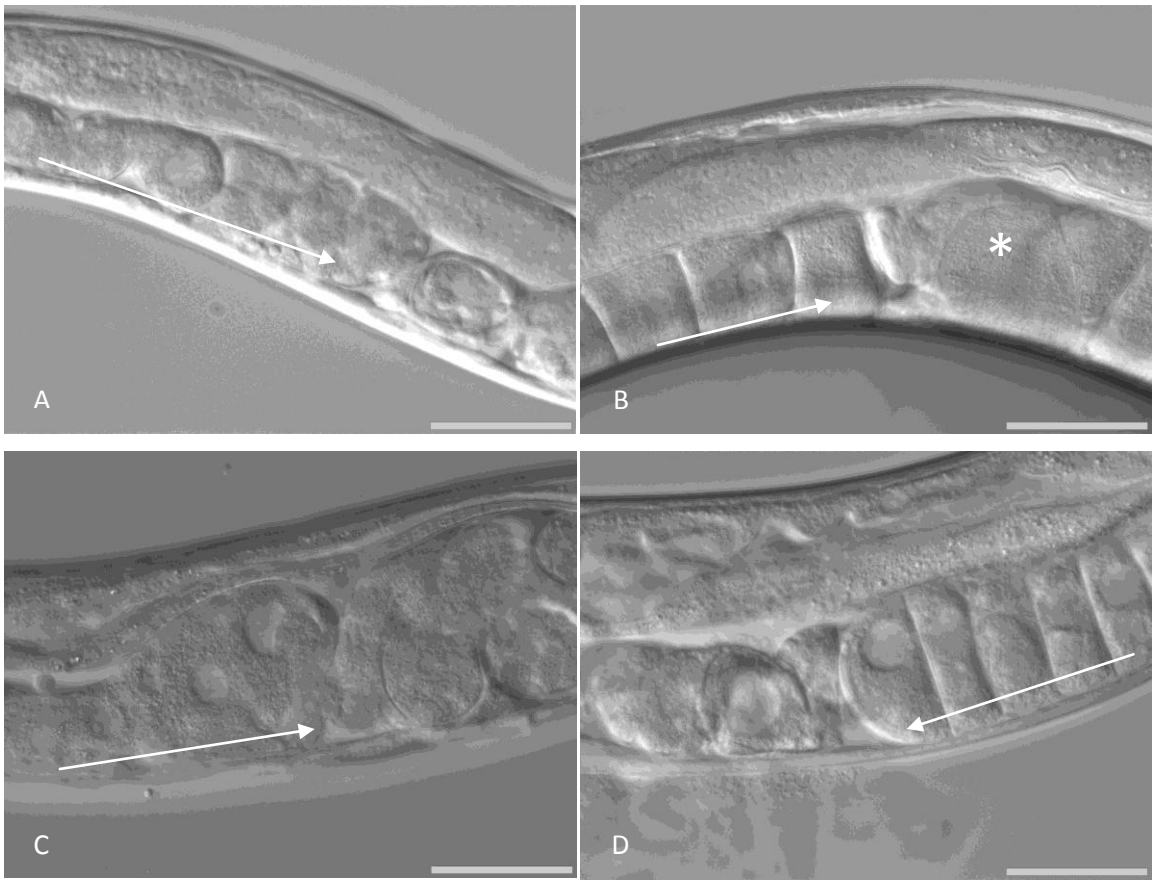

Figure 3.3. Worms fed *rpl-20* RNAi bacteria. (A) A wild-type N2 worm displaying compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing rescued sterility with fertilized eggs in the uterus. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A *lfe-2* worm showing rescued sterility with fertilized eggs in the uterus. White bars = 55μm. Arrows indicate proximal gonad with arrow head toward spermatheca. Asterisk indicates egg in utero.

(Figure 3.6). The mutant backgrounds showed a similar pattern with *itr-1 (sy290)* and *lfe-2* worms, presenting a low incidence of sterility compared to the *ipp-5* mutants. *itr-1 (sy290)* mutants that were fed *rpl-36* dsRNA showed only 13% (N=30) of worms to be sterile, while the *ipp-5* mutants were 100% (N=30) sterile. *lfe-2* mutants, like the *itr-1 (sy290)*, showed low sterility in comparison, with only 7% (N=30) of the worms being sterile. Since the sterile phenotype was able to be rescued in both the *lfe-2* and the *itr-1 (sy290)* mutants, there may be a possible role for these ribosomal proteins in the negative regulation of IP_3 in the signaling pathway.

Signaling and Architecture Proteins

Signaling proteins are obviously good candidates to be involved in the IP_3 pathway. Known components of the IP_3 signaling pathway include *plc-3*, *ppk-1*, and *itr-1*, and were looked at using RNAi in N2 and all three mutant backgrounds (Table 3.2). *plc-3* encodes a phospholipase which cleaves PIP_2 into IP_3 and DAG. IP_3 then goes on to bind to its receptor, ITR-1 on the endoplasmic reticulum, which will then release calcium ions into the cytoplasm. The calcium ions then help control contractions of the sheath cells and ovulation of mature oocytes (Yin and others 2004). *ppk-1* encodes a phosphatidylinositol-4-phosphate 5' kinase, which is needed for ovulation and myosin organization (Xu and others 2007).

RNAi was performed using *plc-3* dsRNA producing bacteria (Figure 3.7). Since *plc-3* is a known component of the IP3 signaling pathway, the results were as expected, with high sterility in the N2, *ipp-5*, and *lfe-2* backgrounds and a complete

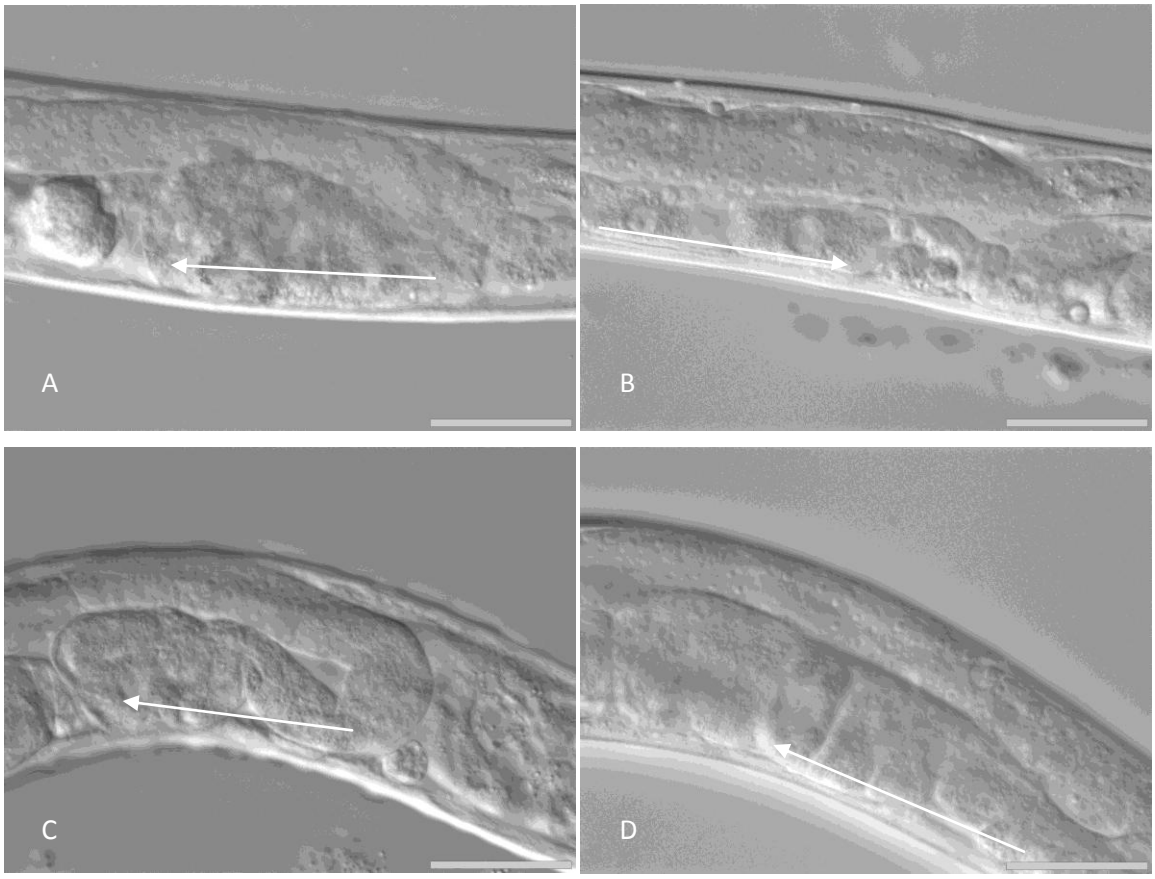

Figure 3.4. Worms fed *rpl-7A* RNAi bacteria. (A) A wild-type N2 worm displaying compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing well formed and well aligned oocytes in the proximal gonad. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A *lfe-2* worm showing well formed and well aligned oocytes in the proximal gonad. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca.

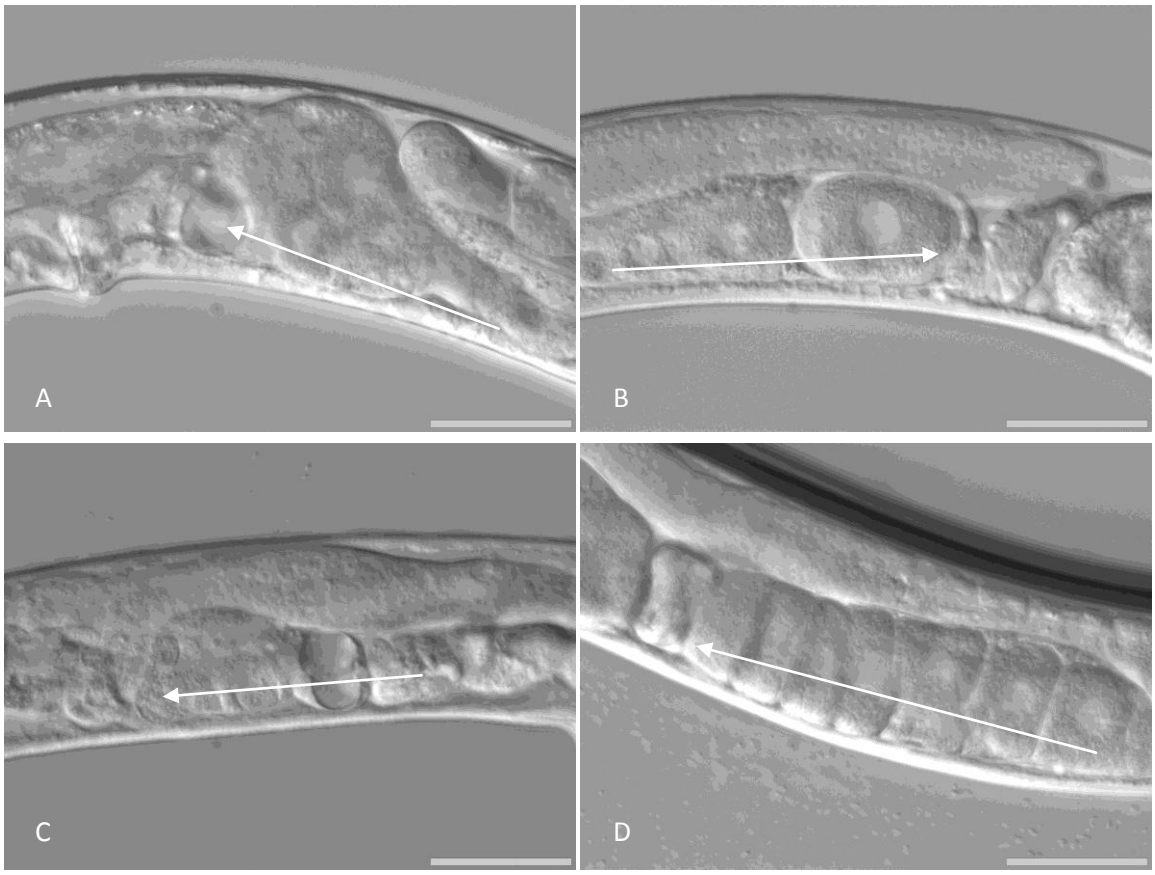

Figure 3.5. Worms fed *rpl-36* RNAi bacteria. (A) A wild-type N2 worm displaying compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing rescued fertility with a fertilized egg in the uterus. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A *lfe-2* worm showing well formed and well aligned proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca.

rescue in the *itr-1 (sy290)* background. The N2 worms were sterile 100% (N=30) of the time as were the *lfe-2* mutants (N=60). The *ipp-5* mutant background was also very high, with 98% (N=60) sterility. The *itr-1 (sy290)* background was able to completely rescue the worms from the *plc-3* RNAi sterile phenotype, showing zero sterile worms (N=30).

When translation of *ppk-1* was eliminated through RNAi, the N2 worms showed a high, 87% (N=30) rate of sterility, while the phenotype was mostly rescued in the *itr-1 (sy290)* background, with a much lower rate of 20% (N=30). The other two mutant backgrounds, *ipp-5* and *lfe-2*, also showed sterility at a much lower rate than the N2, with both only producing sterile worms 17% (N=30 for both) of the time. With RNAi of *itr-1*, another signaling molecule, which acts as a receptor for IP_3, the animal is unable to ovulate because of the lack of calcium release from the ER to produce sheath cell contractions (Figure 3.8). All of the lines used, N2, *ipp-5*, *lfe-2*, and *itr-1(sy290)*, produced 100% sterility in the animals (N=30 for all lines). These results were expected since it was already known that these molecules are involved in the IP_3 signaling pathway.

epi-1 is another gene that encodes a signaling molecule in *C. elegans* (Table 3.2). It codes for a laminin alpha chain protein of the basement membrane and impacts oogenesis through proper gonad organization (Zhu and others 1999; Hall and others 1999). N2 worms which had *epi-1* translation knocked down by RNAi showed 97% (N=30) sterility along with the mutants *ipp-5* and *lfe-2*, which had sterility ratings of 95% (N=60) and 97% (N=60), respectively (Figure 3.9). What was unusual was that the *itr-1 (sy290)* mutant also had a relatively high amount of

Table 3.2. Additional genes causing ≥60% sterility in N2 worms. N = number of animals.

Locus	Function	Reference	Category	% Sterile (N)			
				N2	*itr-1 (sy290)*	*ipp-5*	*lfe-2*
ubq-2	Ubiquitin/60S ribosomal protein L40 fusion	Jones 1995	Ribosomal	77 ± 7.7 (30)	10 ± 4.1 (30)	100 (60)	32 ± 6.5 (60)
epi-1	Laminin alpha chain, oogenesis	Zhu 1999	Cellular Signaling	97 ± 3.1 (30)	77 ± 6.4 (30)	95 ± 2.8 (60)	97 ± 2.2 (60)
plc-3	Phospholipase C; Cleaves PIP2	Yin 2004	Cellular Signaling	100 (30)	0 (30)	98 ± 2.1 (60)	100 (60)
itr-1	Inositol 1,4,5-triphosphate receptor	Clandinin 1998	Cellular Signaling	100 (30)	100 (30)	100 (30)	100 (30)
Talin	Cell migration and contractility; Co-localizes with integrin in focal adhesions	Xu 2005	Cellular architechture	100 (30)	100 (30)	100 (60)	100 (60)
ppk-1	Intense sheath cell contractions, spermatheca dilation	Xu 2007	Cellular Signaling	87 ± 6.5 (30)	20 ± 7.3 (30)	17 ± 6.9 (30)	17 ± 6.9 (30)

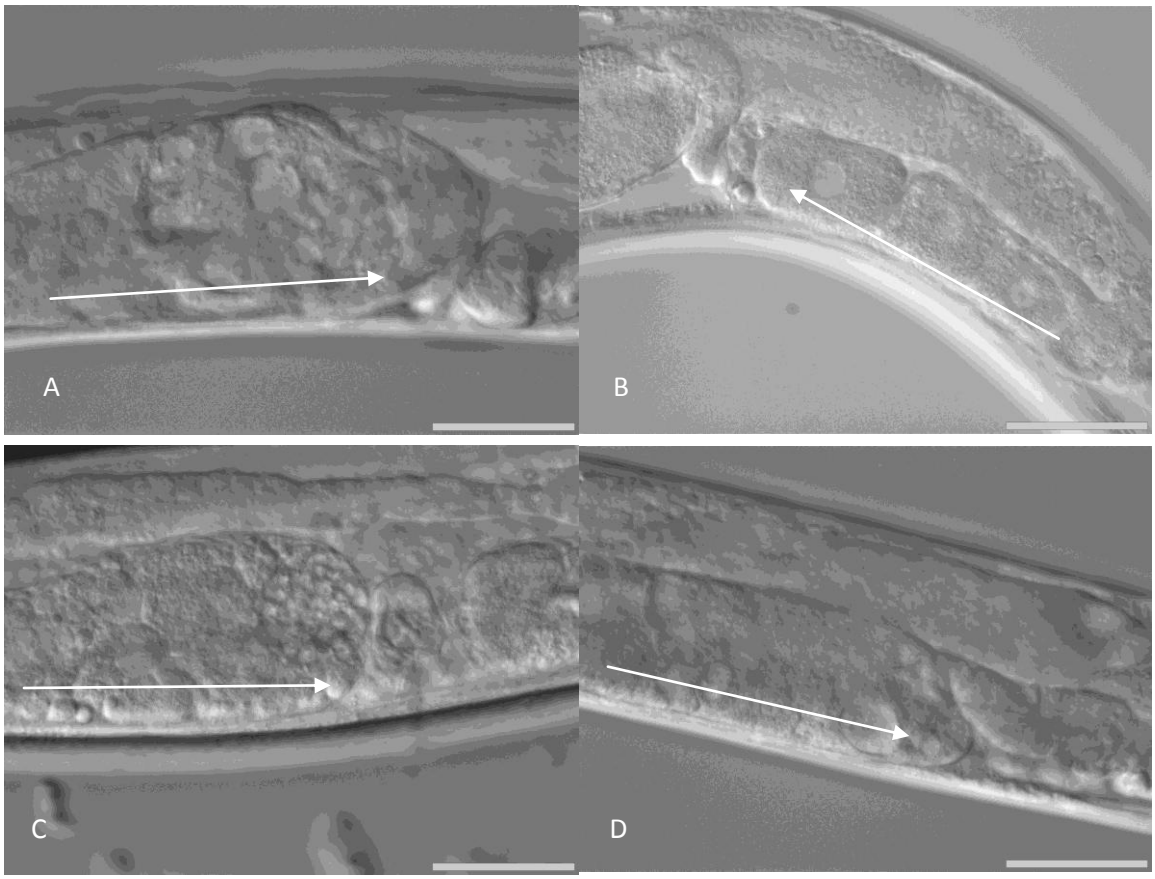

Figure 3.6. Worms fed *plc-3* RNAi bacteria. (A) An N2 worm displaying compacted proximal oocytes. (B) An *itr-1 (sy290)* worm displaying well formed and aligned oocytes. (C) An *ipp-5* worm displaying compacted proximal oocytes. (D) A *lfe-2* worm displaying compacted proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca.

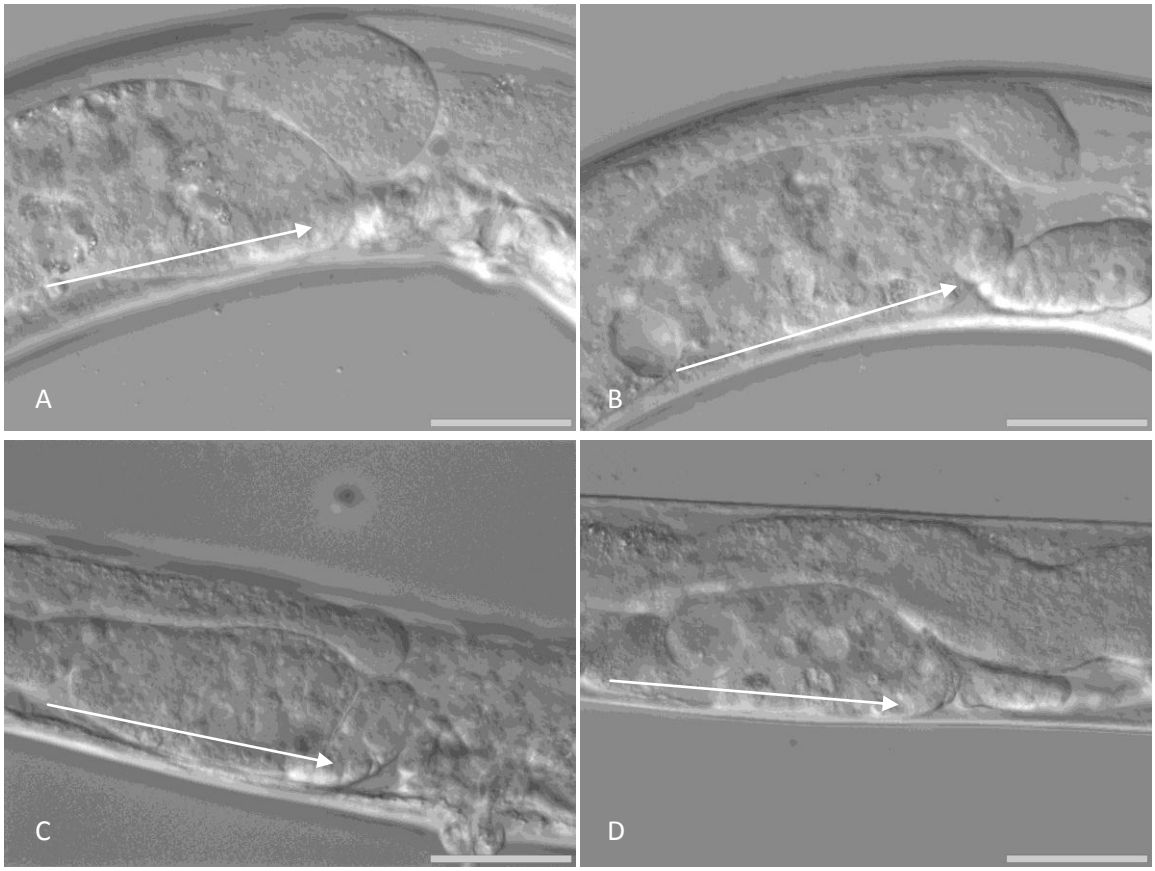

Figure 3.7. Worms fed *itr-1* RNAi bacteria. (A) N2 worm showing compacted proximal oocytes. (B) Worm with an *itr-1 (sy290)* background showing compacted proximal oocytes. (C) Worm with an *ipp-5* background showing compacted proximal oocytes. (D) Worm with *lfe-2* background showing compacted proximal ooctes. White bars = 55μm. Arrows indicate proximal gonad with arrow head toward spermatheca.

sterile worms, with 77% (N=30) not producing any progeny. So, it seems that *epi-1* is necessary in these sheath cells for proper ovulation of the oocytes.

Talin is a cytoskeletal protein that localizes mostly at focal adhesions and dense bodies and can bind to many other proteins in the cytoskeleton including β integrin, actin filaments, and vinculin (Calderwood and others 1999)(Table 3.2). Some studies have shown that talin is involved in the migration of the distal tip cells (DTC) for the appropriate formation of the *C. elegans* gonads and is also necessary for the development of mature oocytes (Cram, Clark, Schwarzbauer 2003). Talin loss-of-function worms also showed a phenotype that was uncoordinated due to the need of talin's interaction with F-actin for proper muscle contraction (Cram, Clark Schwarzbauer 2003). Since talin is involved in both the formation of the actin cytoskeleton and with muscle contraction downstream from the IP_3 pathway, it was not a surprise that the worms were sterile when the translation of talin was eliminated through RNAi. All four worm lines used, N2, *itr-1 (sy290)*, *ipp-5* and *lfe-2*, had 100% (N=30 for N2, *itr-1(sy290)*; N=60 for *ipp-5, lfe-2*) of the worms sterile when RNAi of talin was induced (Figure 3.10).

The last gene looked at that caused significant sterility in the N2 is *ubq-2* (Figure 3.11). *ubq-2* is a gene that is involved in protein synthesis as well as protein degradation. It is also one of only two genes that can produce a full ubiquitin molecule in *C. elegans* (Jones and others 1995). In this study, RNAi knock down of *ubq-2* caused 77% (N=30) of N2 worms to be sterile, while the *itr-1 (sy290)* mutants were almost completely rescued again with only 10% (N=30) resulting in a sterile

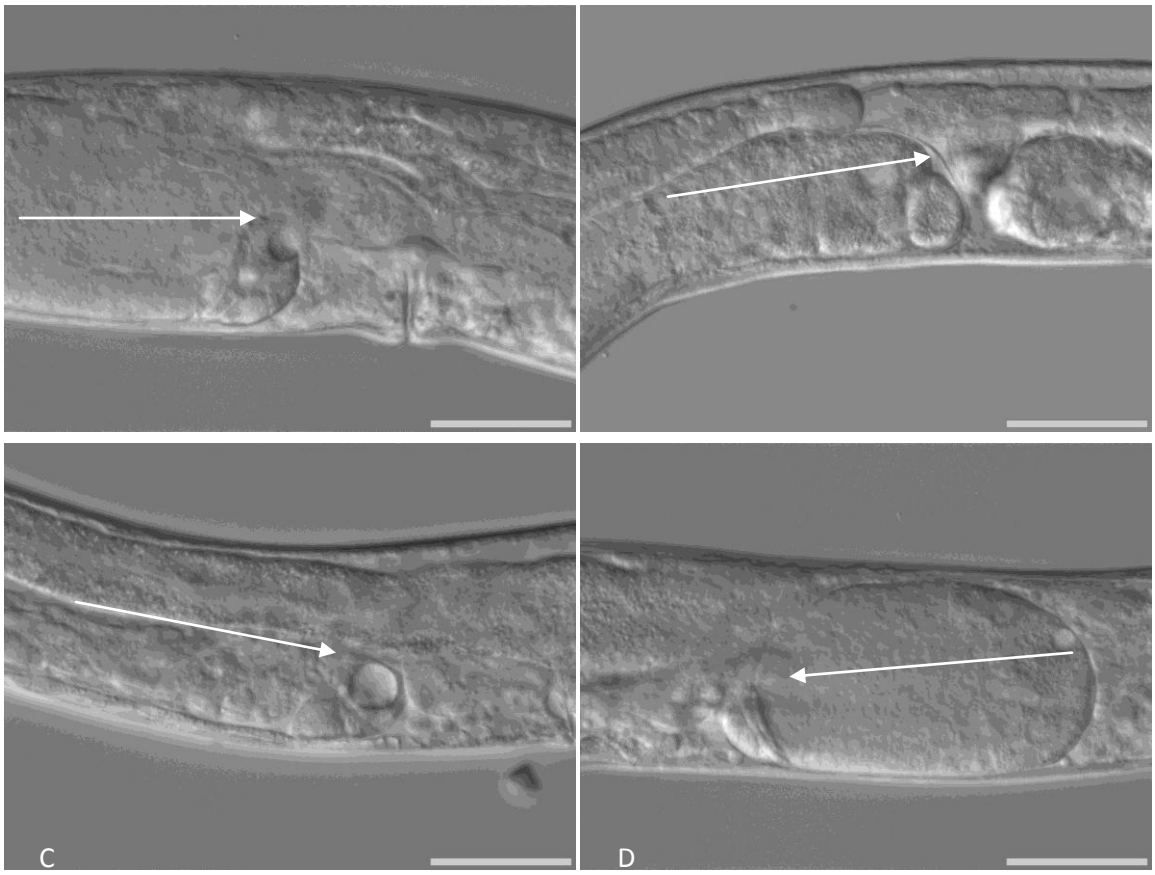

Figure 3.8. Worms fed *epi-1* RNAi bacteria. (A) N2 worm showing abnormally shaped gonad and compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing compacted proximal oocytes. (C) An *ipp-5* worm showing abnormally shaped gonad and compacted proximal oocytes. (D) A *lfe-2* worm showing abnormally shaped gonad and compacted proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca.

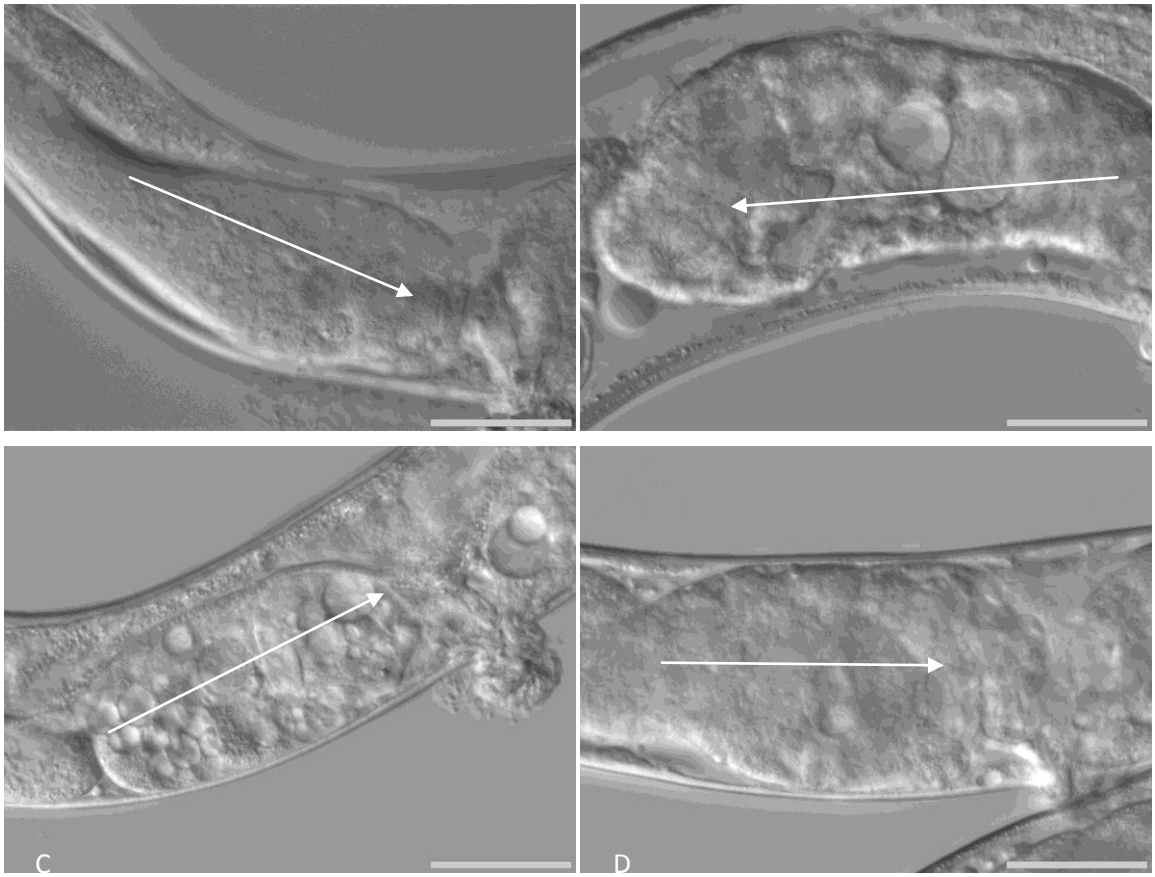

Figure 3.9. Worms fed *Talin* RNAi bacteria. (A) N2 worm showing abnormally shaped gonad and compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing abnormally shaped gonad and compacted proximal oocytes. (C) *ipp-5* worm showing abnormally shaped gonad and compacted proximal oocytes. (D) *lfe-2* worm showing abnormally shaped gonad and compacted proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca.

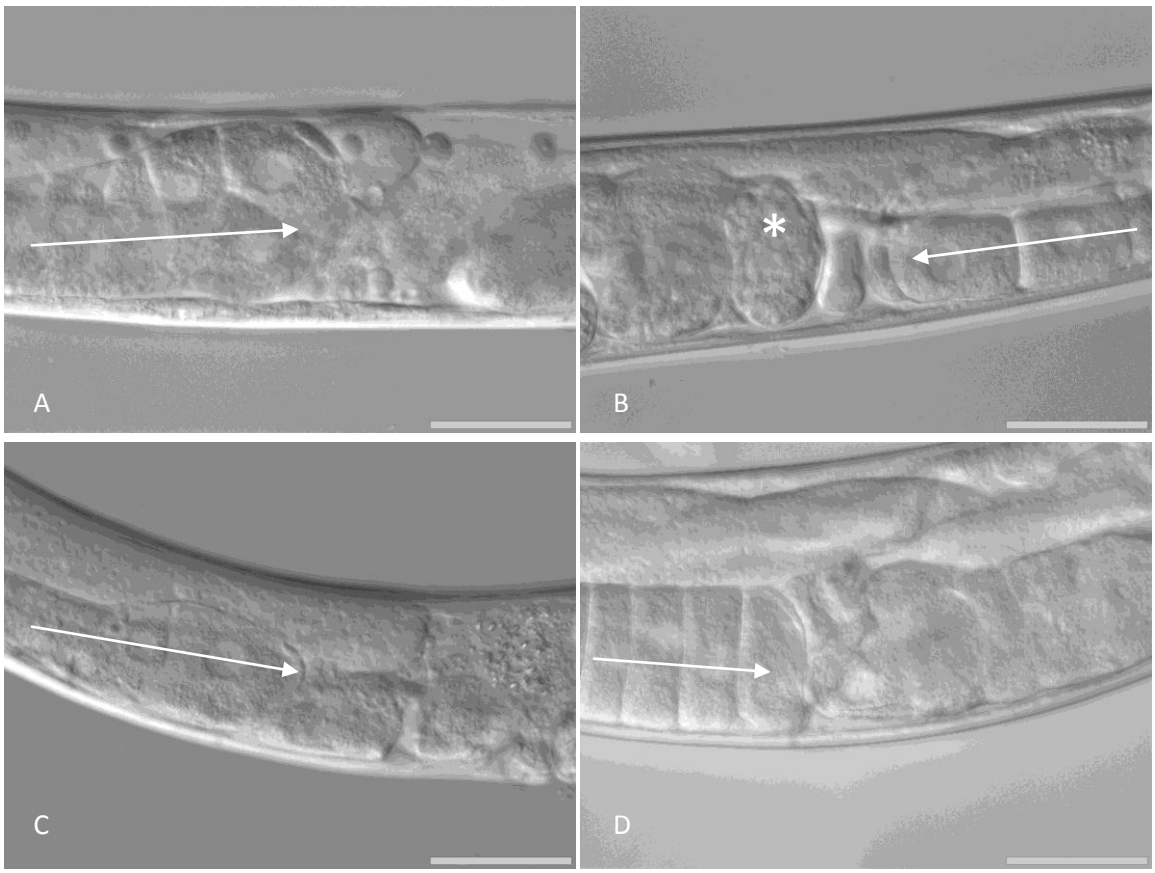

Figure 3.10. Worms fed *ubq-2* RNAi bacteria. (A) N2 worm showing compacted proximal oocytes. (B) An *itr-1 (sy290)* worm showing well formed and aligned proximal oocytes. (C) An *ipp-5* worm showing compacted proximal oocytes. (D) A *lfe-2* worm showing well formed and aligned proximal oocytes. White bars = 55µm. Arrows indicate proximal gonad with arrow head toward spermatheca. Asterisk represents egg in utero.

phenotype. However, it seems quite evident that *ubq-2* is involved in the IP3 pathway somehow since the *ipp-5* mutant showed 100% (N=60) sterility with the elimination of talin and the *lfe-2* mutant produced sterile progeny at 32%(N=60).

CHAPTER FOUR

Discussion and Conclusions

In this study, the role of *lfe-2* and *ipp-5* in the IP_3 signaling pathway were investigated by using RNA interference to eliminate translation of certain genes. The genes that were removed had previously been shown to cause sterility in 60% of N2 worms, but had rescued sterility in worms with an *itr-1 (sy290)* background (Miles unpublished data). Here, these genes, mostly encoding ribosomal proteins, were eliminated in worms with a background of either *ipp-5* or *lfe-2* and it was found that many of the ribosomal genes had rescued sterility in the *lfe-2* background, but remained sterile in the *ipp-5* background.

One explanation for this could be that *lfe-2* is expressed solely in the adult spermatheca (Clandinin and others 1998), as is *ipp-5* (Bui and Sternberg 2002). In the *ipp-5* mutants, IP_3 signaling seems to be higher and the spermatheca dilates and extends farther than in the wild type animal. This allows not only the matured proximal oocyte to be ovulated, but also the second oocyte to be ovulated as well, even though it has not undergone maturation. Because of this it seems that *ipp-5* is necessary to prevent hyperextension of the spermatheca through negative regulation of IP_3 signaling (Bui and Sternberg 2002).

Studies of IP_3 metabolism in *Xenopus* oocytes have shown that with lower concentrations of IP_3 along with high concentrations of calcium, IP_3 tends to be metabolized by the IP_3 kinase (LFE-2), whereas if IP_3 concentrations are high, the phosphatase (IPP-5) more readily degrades the IP_3 (Sims and Albritton 1996). If

this is the case in *C. elegans* ovulation, then in the absence of IPP-5, it is possible that the IP_3 concentration becomes quite high and is unable to be metabolized by LFE-2 which results in the spermatheca being dilated for an extended time and allowing two oocytes to be ovulated.

When RNA interference was used to eliminate expression of certain protein synthesis proteins, the outcome was rescued sterility in the *lfe-2* background and continued sterility in the *ipp-5* background. These resulting double mutations could cause negative feedback of the IP_3 signaling due to elevations in IP_3 concentrations, causing the sterile phenotypes seen in the *ipp-5* background. The rescued sterility in the *lfe-2* background may be possible because of the necessity of IPP-5 to dilate the spermatheca to the appropriate extension to allow ovulation of a single oocyte. Since there is not an excess of IP_3, the pathway does not show the negative feedback or sterility that loss of *ipp-5* causes.

Elimination of these protein synthesis proteins could also cause undue stress upon the endoplasmic reticulum. In general, as ribosomes translate secretory proteins, they are immediately transported into the ER where chaperones will be present to assist in their proper folding. If certain protein synthesis proteins are no longer expressed, then translation may not occur properly and newly synthesized proteins may not be transported into the ER. As seen here, without proper proteins being produced, oocytes in the *C. elegans* gonads are not able to be ovulated properly.

Another explanation for this phenomenon could be that *ipp-5* is not solely expressed in the spermatheca, but also required in the myoepithelial sheath cells,

while *lfe-2* is only expressed in the spermatheca. Because *lfe-2* is expressed only in the spermatheca, mutants lacking *lfe-2* would have an increase in IP_3 in the spermatheca only, allowing normal IP_3 concentrations in the sheath cells, and therefore allowing normal ovulation of the mature oocytes. However in the *ipp-5* mutants, IP_3 would increase in both the spermatheca and the sheath cells simultaneously. This could cause release of numerous premature oocytes due to failure of the spermatheca to close as well as paralyzed sheath cells. With the RNAi of the ribosomal genes in the *ipp-5* background it seems there are extreme defects that cannot be suppressed, with sterility possibly caused by "spill over" of premature oocytes instead of actual ovulation defects. With *lfe-2* only expressed in the spermatheca, RNAi in these mutants is able to be suppressed in the case of the ribosomal proteins because it does not affect the contraction of the sheath cells.

The results seen with the elimination of some of the cellular architechture and signaling proteins showed that in most cases, neither *ipp-5* nor *lfe-2* backgrounds were able to rescue the sterility seen in the N2. Talin showed some of the most severe effects on the worms' fertility, causing sterility in all of the mutant backgrounds observed. Talin is a protein that is involved in cell migration and cell contractility and also interacts with integrin at focal adhesions (Xu 2005). Talin has been shown to help direction of distal tip cell (DTC) migration so that the gonads form properly (Cram, Clark, Schwarzbauer 2003). In the mutant backgrounds used here, loss of talin caused poor gonad morphogenesis, with several worms showing gonads that did not make the proper U-shape turn. It does not seem that talin is

directly involved in the IP_3 pathway, as even the *itr-1 (sy290)* mutant background could not rescue sterility.

plc-3 and *itr-1* are both directly involved in the IP_3 pathway, but the sterility that the loss of the genes caused could not be rescued in the *ipp-5* and *lfe-2* mutant backgrounds as was seen in with the *itr-1 (sy290)* background. With loss of *itr-1* completely, there is no receptor on the ER for the IP_3 to bind to, leaving the calcium ions trapped and resulting in fewer sheath cell contractions. With loss of *plc-3*, PIP_2 is not able to be cleaved into DAG and IP_3. RNAi of *plc-3* caused sterility in all lines except *itr-1 (sy290)* where the IP_3 receptor is constitutively active.

Future Directions

RNAi of different genes using mutant backgrounds like *ipp-5* and *lfe-2* gave further insight into the IP_3 signaling pathway and its involvement in *C. elegans* ovulation. Of the genes that had an effect on the negative regulation of IP_3, the majority of them were ribosomal genes which are more ubiquitously expressed. RNAi in the *lfe-2* mutants was able to be suppressed, but this was not so in the *ipp-5* mutants. It would be of use to do studies focused on localization of these genes using fusion proteins like GFP to gain a greater understanding of exactly what tissues they are expressed.

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